

Efficacy of a *Streptococcus iniae* modified bacterin delivered using Oralject™ technology in Nile tilapia (*Oreochromis niloticus*)

Craig A. Shoemaker^{a,*}, Grant W. Vandenberg^c, André Désormeaux^c,
Phillip H. Klesius^a, Joyce J. Evans^b

^a Aquatic Animal Health Research Laboratory, United States Department of Agriculture–Agricultural Research Service, Auburn, AL, USA

^b Aquatic Animal Health Research Laboratory, United States Department of Agriculture–Agricultural Research Service, Chestertown, MD, USA

^c PerOs Systems Technologies, Inc., St-Nicolas, Quebec, Canada

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Abstract

This study evaluated the potential to orally deliver a previously developed *Streptococcus iniae* vaccine in tilapia using Oralject™ technology. This technology was developed to administer bioactive compounds to monogastric animals, and has been shown to be effective for delivery of a variety of antigens in numerous fish species. Two different formulations containing two doses of vaccine (four treatments) were fed to tilapia (4 tanks of 25 fish each) for 1 (Oralject-1 and Oralject-2 each containing 2×10^9 cells/g of feed) day (am and pm to satiation) or 5 (Oralject-1 and -2 each containing 2×10^8 cells/g of feed) days (once daily to satiation). The incorporated vaccine was a patented lyophilized modified bacterin (US Patent No. 6,379,677 B1). A positive control treatment [intraperitoneally (i.p.) injected *S. iniae* vaccine] and a negative control treatment (i.p. injection of tryptic soy broth, TSB) were included. Mean percent intake indicated that tilapia fed for 1 day (twice to satiation) the Oralject-1 consumed significantly ($P < 0.05$) more feed than fish fed Oralject-2 (4.05% vs. 3.21%, respectively). Fish fed for 5 days either commercial feed or Oralject-1 or -2 also showed differences in feed intake; on most days, fish consumed significantly less ($P < 0.05$) Oralject-2 (~1%) than the commercial diet or Oralject-1 (~2.5%). Tilapia were challenged 23 days post-vaccination by i.p. injection of 1×10^6 CFU *S. iniae*/fish. Mean percent mortality was $47.5 (\pm 7.5)$ in the TSB-immunized challenged tilapia and was significantly higher ($P < 0.001$) than in all immunized groups. No mortality occurred in the i.p.-vaccinated tilapia. Mortality ranged from 17.5 to 31.25 in the Oralject™ treatments. Relative percent survival was 100% in the i.p.-vaccinated tilapia and 63.1% in the most effective Oralject-vaccine-treated group. The results suggest that oral delivery of the lyophilized *S. iniae* vaccine using Oralject™ technology provided protection against streptococcal disease. These data validate an initial proof-of-principle for oral vaccination of tilapia using *S. iniae* in the Oralject™ system.

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Keywords: Oralject™; *Streptococcus iniae*; Oral vaccination; Nile tilapia

1. Introduction

Streptococcus iniae is one of the most significant Gram-positive pathogens in wild and cultured fish species worldwide. Estimates of losses in the US alone exceed \$10 million annually (Shoemaker and Klesius,

* Corresponding author. Tel.: +1 334 887 3741; fax: +1 334 8872983.

E-mail address: cshoemaker@ars.usda.gov (C.A. Shoemaker).

1997). Efficacious vaccines [bacterins or modified bacterins (i.e., containing extracellular products)] have been developed against *S. iniae* for delivery by injection (Eldar et al., 1997; Klesius et al., 1999; Klesius et al., 2000; Klesius et al., 2002). Attempts at immersion vaccination using these killed *S. iniae* vaccines have been unsuccessful. The injected vaccines while being effective are labor-intensive to deliver and induce stress (i.e., fish have to be handled). A potential mass delivery strategy is oral administration via feed (Vandenberg, 2004). Ease of delivery (i.e., feeding) would enable mass vaccination of large numbers of fish in hatcheries, ponds and even the environment.

Other bacterial vaccines for fish have been successfully delivered by oral administration; however, the efficacy of the vaccines has not been as good as by parenteral injection (Ellis, 1988; Newman, 1993). Recently, Romalde et al. (2004) reported the use of alginate microparticles containing formalin-killed *Lactococcus garviae* as an oral vaccine. The best protective rate based on relative percent survival (RPS) using this method was 50%. Romalde et al. (2004) were able to demonstrate that fish immunized with an aqueous vaccine by injection and boosted via oral delivery at 4 months were protected (RPS=87%). The aqueous vaccine alone failed to provide significant protection after the third month (RPS=40%) following intraperitoneal (i.p.) challenge (Romalde et al., 2004). A similar protective effect was suggested following oral re-immunization after initial immersion vaccination of European eels against *Vibrio vulnificus* (Esteve-Gassent et al., 2004). The oral vaccine was prepared by the addition of bacterial antigen to feed without a carrier or protective coating.

Vandenberg et al. (2003) proposed a novel delivery strategy (Oralject™) for oral vaccination of monogastric animals. The Oralject™ technology relies on the temporary reduction of the digestive processes by administration of anti-proteases and membrane permeability enhancers in combination with the vaccine. This approach permits the vaccine (i.e., antigen) to escape digestive hydrolysis and have enhanced vaccine component uptake (Vandenberg, 2004).

The objective of this study was to determine the efficacy of *S. iniae* modified bacterin incorporated in fish feed using Oralject™ technology¹ to provide

protection against streptococcal disease in Nile tilapia (*Oreochromis niloticus*).

2. Materials and methods

2.1. Fish and feeding

A total of 600 Nile tilapia (*O. niloticus*) with a mean weight of 12.7 grams/fish was utilized in this study. Prior to the study 10 fish were found to be culture negative for *S. iniae* by standard microbiology (Shoemaker et al., 2001). Fish were weighed and divided into four replicate aquaria of 25 fish each for each treatment. Each aquarium was supplied with de-chlorinated city water ($26\pm 2^\circ\text{C}$) at a rate of 0.5 l/min. Fish were acclimated for 1 week prior to treatment and fed at a rate of 2% body weight (BW)/day with Aquamax Grower 400 (Brentwood, MO). After the 7-day acclimation period, fish were fasted for 36 h. After feed withdrawal for 36 h, fish in group A were injected intraperitoneally (i.p.) with 100 μl of sterile tryptic soy broth (TSB) (Table 1). Fish from group B were injected i.p. with lyophilized *S. iniae* vaccine (Klesius et al., 2000) resuspended in TSB at a rate of 100 μl per fish (equivalent to 4×10^8 CFU/fish). The lyophilized *S. iniae* vaccine was prepared by culturing *S. iniae* isolate ARS-60 for 72 h in tryptic soy broth prior to killing with formalin. The *S. iniae* cells were then removed from the culture fluid via centrifugation. After removal, the culture fluid was concentrated (20-fold) via use of a 2-kDa hollow fiber filter. Following sterile filtration (0.2 μm pore size), formalin-killed cells were added back to a final concentration of 4×10^9 cells/ml. Two different Oralject containing vaccine formulations were fed to the other tilapia. Fish from groups C and D were fed for 1 day (am and pm) to satiation with Oralject formulations-1 and -2 containing 2×10^9 cells/g feed, respectively. Fish from groups E and F were fed for 5 days once daily to satiation with Oralject formulations-1 and -2 containing 2×10^8 cells/g feed, respectively. Amount of feed consumed for each group was recorded daily during the 1-day treatment and 5-day treatment. Feed consumed was expressed as a percentage of the initial total weight of the fish in the tank. Following the feeding of the vaccine formulations, all fish were fed Aquamax Grower 400 (Brentwood, MO) at a rate of 2% initial body weight once daily.

2.2. Experimental challenge and antibody determination

All groups of fish (20/tank) were challenged 23 days after final feeding of the orally delivered vaccine.

¹ Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendations or endorsement by the USDA.

Table 1

Mean percent feed intake of control (tryptic soy broth only injection), vaccine injection, Oralject-1 (2×10^8 CFU/g) and Oralject-2 (2×10^8 CFU/g) fed tilapia for the 5-day feeding period

Treatment	Mean percent feed intake (\pm S.E.) ¹				
	Day 1	Day 2	Day 3	Day 4	Day 5
Negative control	2.17 \pm 0.04 ^{a2}	2.65 \pm 0.04 ^a	2.81 \pm 0.03 ^a	2.80 \pm 0.07 ^a	2.63 \pm 0.04 ^a
Injected vaccine	1.97 \pm 0.05 ^b	2.62 \pm 0.05 ^a	2.83 \pm 0.06 ^a	2.76 \pm 0.02 ^a	2.75 \pm 0.06 ^a
Oralject-1	2.39 \pm 0.05 ^c	1.56 \pm 0.11 ^b	3.12 \pm 0.05 ^b	2.67 \pm 0.05 ^a	3.03 \pm 0.05 ^b
Oralject-2	2.30 \pm 0.10 ^{a,c}	1.15 \pm 0.05 ^c	1.14 \pm 0.01 ^c	1.18 \pm 0.05 ^b	1.13 \pm 0.04 ^c

¹ Mean percent feed intake is represented by the total feed intake for the four replicate tanks (25 fish/tank) for each treatment that was fed to satiation for the 5-day vaccine feeding period. The feed intake for the control and i.p. injected treatment is the amount of commercial ration consumed.

² Means analyzed by one-way analysis of variance using the GLM procedure and Duncan's multiple range test to determine significance at $P < 0.05$ (SAS 2002). Different letters indicate significant differences in feed intake for that day (i.e., in the column).

Briefly, *S. iniae* (ARS-60) was grown in TSB for 24 h at 28 °C with shaking on an orbital shaker (100 revolutions per minute) (Klesius et al., 2000). Fish were i.p. injected with 1×10^6 CFU/fish. Following challenge, mortality was monitored and recorded daily for 21 days. Cumulative percent mortality and relative percent survival (Amend, 1981) were calculated. Freshly dead fish were cultured for the presence of *S. iniae* to confirm cause of mortality using standard bacteriology procedures (Shoemaker et al., 2001).

Prior to and following challenge, 2–3 fish/tank were bled via the caudal vasculature (the fish were killed by an overdose of tricaine methanesulfonate 200 mg/l) for the determination of antibody level against *S. iniae* using the method described by Shelby et al. (2002). Briefly, enzyme-linked immunosorbent assay (ELISA) plates were coated for 1 h at 25 °C with 100 μ l *S. iniae* antigen in carbonate buffer (CB), that was obtained following sonication and size-exclusion chromatography of *S. iniae* (a 1:10 dilution in CB) of the initial fraction which represented the highest molecular weight fraction was used). Plates were washed with phosphate-buffered saline (PBS) and then blocked with 3% bovine serum albumin in CB for 1 h. Following blocking, plates were again washed with PBS (three times). Tilapia serum was then added at a 1:20 dilution in phosphate-buffered saline +0.05% Tween-20 (PBS-T). Serum was incubated for 30 min at 25 °C and then plates washed 3 times with PBS-T. About 100 μ l of monoclonal anti-tilapia immunoglobulin (Shelby et al., 2002) at a 1:10 dilution in PBS-T was then added to all wells for 30 min. Following washing in PBS-T, 100 μ l of sheep anti-mouse IgG peroxidase conjugate was added and incubated for 15 min. Plates were washed as above and 100 μ l *o*-phenylenediamine (OPD) in urea peroxide buffer was added to each well for 15 min. The reaction was stopped with 50 μ l 3 M H₂SO₄. Serum antibody levels

were based on the optical density (OD) reading of the reaction measured spectrophotometrically at 450 nm. *S. iniae*-positive and -negative sera were included on each plate as assay controls.

2.3. Statistical analysis

Data were analyzed by one-way analysis of variance using the general linear model procedure of Statistical Analysis System (SAS 2002). Duncan's multiple range test was used to compare treatment means for both mortality and antibody OD, differences were considered significant at $P < 0.05$.

3. Results and discussion

Mean percentage feed intake (mean of the four replicate tanks) for the 1 day (am and pm) satiation feeding of Oralject-1 (2×10^9 1 day feeding = $4.05 \pm 0.24\%$) was significantly higher ($P < 0.05$) than for fish fed Oralject-2 (2×10^9 1 day feeding = $3.21 \pm 0.14\%$). Results of the fish fed Oralject-1 (2×10^8 cells/g) and Oralject-2 (2×10^8 cells/g) once daily to satiation for 5 days are presented in Table 1. In general, fish fed the commercial diet and Oralject-1 (2×10^8 cells/g) consumed significantly more ($P < 0.05$) feed than fish fed the Oralject-2 (2×10^8 cells/g) formulation over the 5-day period. Fish fed the Oralject-1 formulation consumed more of this preparation than Oralject-2 regardless of feeding regimen. This observation is probably due to a more favorable palatability of Oralject-1. Mean percent survival ranged from 90% to 97% for all treatment groups prior to challenge. *S. iniae* was not isolated from any of these dead fish.

Mean percent mortality was 47.5% in the TSB-immunized challenged tilapia and was significantly higher ($P < 0.001$) than in all immunized groups (Table 2). No mortality occurred in the i.p.-vaccinated tilapia.

Table 2

Efficacy of different vaccine formulations administered to tilapia following intraperitoneal (i.p.) injection or oral delivery

Treatment	Dose	No. dead/ No. total ¹	Mean % mortality (\pm S.E.) ²	Relative percent survival (RPS)
Negative control (sterile tryptic soy broth)	100 μ l	38/80	47.50 (\pm 7.5) ^a	–
Injected vaccine (modified bacterin)	100 μ l (4×10^8 CFU/fish)	0/80	0.00 (\pm 0.0) ^b	100.0
Oralject-1 (1-day feeding)	2×10^9 CFU/g feed	14/80	17.50 (\pm 3.2) ^c	63.1
Oralject-2 (1-day feeding)	2×10^9 CFU/g feed	18/80	22.50 (\pm 5.9) ^c	52.6
Oralject-1 (5-day feeding)	2×10^8 CFU/g feed	17/80	21.25 (\pm 3.1) ^c	55.3
Oralject-2 (5-day feeding)	2×10^8 CFU/g feed	25/80	31.25 (\pm 3.8) ^c	34.2

Significant difference is indicated by different superscript letters.

¹ Total is represented by four replicate tanks of 20 fish each. Fish were challenged 23 days post-immunization by i.p. injection with 1×10^6 CFU/ml of *S. iniae* and monitored for 21 days post-challenge.² Means analyzed by one-way analysis of variance using the GLM procedure and Duncan's multiple range test to determine significance at $P < 0.001$ (SAS 2002).

Mortality ranged from 17.5 to 31.25 in the Oralject treatments and was not significantly different among Oralject treatments. Relative percent survival was 100% in the i.p.-vaccinated tilapia, 63.1% and 55.3% in the Oralject-1 formulations fed for 1 and 5 days, respectively. Relative percent survival in the Oralject-2 formulations fed for 1 or 5 days were 52.6% and 34.2%, respectively. The results of this study demonstrate that oral delivery of lyophilized *S. iniae* modified bacterin using Oralject technology provided protection against streptococcal disease following i.p. challenge. Intraperitoneal challenge may not be the appropriate challenge procedure to fully access the protective effect of oral immunization because it bypasses the mucosal immune compartment (Romalde et al., 2004). Romalde et al. (2004) demonstrated an RPS of 50% in rainbow trout immunized orally with alginate microparticles containing *L. garviae* antigens. They suggested that this method be used only as a booster-immunization method to enhance parenteral vaccination longevity. We also employed the i.p. injection challenge method in this study due to the fact it is reproducible for *S. iniae* in tilapia (Klesius et al., 2000; Shelby et al., 2003). Immersion challenge of tilapia results in low levels of mortality (5–35%) even following administration of high numbers of *S. iniae* for a long exposure (Shoemaker et al., 2000).

A humoral antibody response was noted after immunization with the i.p.-injected vaccine and the Oralject-delivered vaccine formulations measured by indirect ELISA (Table 3). Following challenge, no difference was noted in the ELISA OD between the groups (Table 3). Results of other *S. iniae* bacterin studies have demonstrated weak antibody responses following parenteral injection (Eldar et al., 1995; Eldar et al., 1997; Whittington et al., 2003) with or without a subsequent

increase in antibody level post-challenge (Whittington et al., 2005). Shelby et al. (2003) demonstrated by passive immunization that antibody was responsible for protection against *S. iniae*. Antibody OD also correlates with agglutinating antibody titers measured in tilapia (Shelby et al., 2001; Shelby et al., 2002) and hybrid striped bass (*Morone chrysops* \times *Morone saxatilis*) (Shelby et al., 2004). More recently, Vervarcke et al. (2005), using African catfish (*Clarias gariepinus*), demonstrated that the immune response is compartmentalized (i.e., mucosal and systemic immunity). This has been suggested previously in other fish and tilapia species (Jenkins et al., 1994; Cain et al., 2000; Grabowski et al., 2004). Lin et al. (2000) have suggested that oral immunization stimulates mucosal immunity directly, whereas injection stimulates a systemic response with a subsequent cutaneous mucosal response in dab (*Limanda limanda*). In another tilapia species (*Oreochromis mossambicus*), Jenkins et al. (1994) delivered a protein antigen enterically and

Table 3

Mean serum indirect ELISA antibody optical density (OD) prior to challenge (23 days post-vaccination) and 21 days post-challenge with *S. iniae*

Treatment	Post-vaccination OD (\pm S.E.) ¹	Post-challenge OD (\pm S.E.)
Negative control	0.11 (\pm 0.01) ^a	0.32 (\pm 0.03)
Injected vaccine	0.30 (\pm 0.03) ^b	0.34 (\pm 0.03)
Oralject-1 (1-day feeding)	0.20 (\pm 0.02) ^c	0.36 (\pm 0.03)
Oralject-2 (1-day feeding)	0.24 (\pm 0.04) ^{b,c}	0.32 (\pm 0.03)
Oralject-1 (5-day feeding)	0.21 (\pm 0.03) ^c	0.32 (\pm 0.05)
Oralject-2 (5-day feeding)	0.17 (\pm 0.03) ^{a,c}	0.39 (\pm 0.03)

¹ Significant difference in mean antibody optical density (OD) as determined by ELISA (Shelby et al., 2002) at a 1:20 serum dilution post-vaccination or post-challenge is indicated by different superscript letters. Mean antibody OD was determined from at least 6 fish for each treatment post-vaccination and post-challenge.

demonstrated the presence of antibodies in the plasma, bile and cutaneous mucus. Shoemaker et al. (2005) recently demonstrated in channel catfish (*Ictalurus punctatus*) the presence of cutaneous antibodies following parenteral injection and showed that this response correlated with humoral antibody. Although we did not measure the cutaneous immune response in the present study, the observed protection may be due in part to the stimulation of the mucosal response and thus, initiation of immune memory due to stimulation of the gut associated lymphoid tissue (Rombout et al., 1986). Oral delivery of the *S. iniae* modified bacterin using the Oralject™ technology will provide a non-stressful, less labor-intensive and economical method of mass immunization.

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